IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of.:		§		
		§		
	Yoram Reiter	§		
		§		
Serial No.:	10/510,229	§		
		§		
Filed:	10/13/2004	§	Group Art Unit:	1648
		§		
For:	ANTIGEN-PRESENTING COMPLEX-BINDING COMPOSITIONS AND USES THEREOF	§		
		§		
		§	Attorney Docket:	**28429**
		§		
Examiner:	Lucas, Zachariah	§		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF VINCENZO CERUNDOLO UNDER 37 CFR 1.132

I am presently employed as a Principle Investigator where I am a Professor of Immunology at the University of Oxford and Associate Director of the Medical Research Council Human Immunology Unit. I received my Ph.D. degree from the University of Padua, worked as a post-doctoral fellow in the laboratory of Professor Alain Townsend, where I characterized some of the mechanisms controlling the antigen processing and presentation pathways.

My research focuses on the analysis of tumor specific immune response and on the development of novel vaccination strategies. Since the beginning of my career, I have published 170 scientific articles in highly regarded journals and books, and have presented my achievements at many international scientific conferences.

I am a Fellow of the Royal College of Pathologists, and was awarded the Cancer Research Institute, USA Investigator Award. I was elected Fellow to The Academy of Medical Sciences in 2002.

In re Application of: Yoram Reiter
Serial No.: 10/510,229
Filed: 10/13/2004
Office Action Mailing Date: 01/24/2007

Examiner: Lucas, Zachariah
Group Art Unit: 1648
Attorney Docket: 28429

I am considered to be an expert in the field of Immunology.

I have read the Office Action issued with respect to the above-identified patent application.

In this Office Action, the Examiner rejected claims 141-149, 151-155, 158 and 159 under 35 U.S.C. §103(a) as being unpatentable over Reiter (PNAS 94:4631-4636, 1997), further in view of the teachings Andersen et al., (WO 97/02342) who generated antibodies against mouse MHC-peptide complexes but contemplated more. Particularly, the Examiner states that the teachings of Andersen (1997) and Reiter (1997) could be used by any person skilled in the art to generate the antibodies against human MHC-peptide complexes as in the present application by Yoram Reiter.

Being an expert in the field of Immunology, I, Vincenzo Cerundolo, hereby state that the claimed invention, essentially a method of killing or damaging target human cells expressing or displaying a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen using antibodies capable of specifically binding such a complex, is novel and non-obvious over the cited references in that it satisfies a long-felt need which was recognized, persistent and not solved by others at the time of filing of the claimed invention.

I provide herewith documented evidence showing that for more than 15 years prior to the filing date of the invention discussed and claimed in the above-identified patent application, research groups have all failed in generating T-cell receptor (TCR)-like antibodies such as antibodies capable of binding human antigen-presenting molecules and an antigen derived from a pathogen, let alone using them for killing virus infected human cells. In evidence for this I provide herewith several experimental papers all showing failure to obtain TCR-like antibodies for killing virus infected human cells.

Thus, Tamminen WL et al. (Eur. J. Immunol. 1987, 17:999-1006; see, in particular, Abstract; attached herewith) failed to obtain antibodies against MHC-viral peptide complexes.

In addition, Rubin B., et al., 1989 (Res. Immunol. 140:67-74; attached herewith) tested more than 1500 antisera but could not find any antibodies specific for complexes of MHC-insulin peptide (see Page 71, middle paragraph in Rubin et al.).

Chames P., et al., (PNAS, 2000, 97:7969-7974, attached herewith; see in particular Page 7970, left column, first paragraph), state that although highly desired for therapeutic applications, selection of antibodies that recognize MHC-peptide complexes is a difficult task. Chames et al. attempted to identify an antibody with specificity against a human MHC-peptide complex and identified one phage clone displaying Fab G8 which recognized HLA-A1-MAGE-A1 (a melanoma peptide) but not HLA-A1-MAGE-A3 complexes. However, when the soluble G8 Fab fragment, which was purified from the phage clone, was reacted with the target complex, the authors could not find conditions that eluted the antibody without also dissociating the β2m from the HLA-A1 heavy chain, thus, they failed to obtain a TCR-like antibody with specificity to human MHC-peptide complexes (see Page 7972, left column, second column in Chames et al.) which can be used in therapy. In addition, when the purified Fab G8 was immobilized to a surface through its hexahistidine tag, the bound antibody was devoid of affinity sufficient to kill target cells *in vivo*. Moreover, the purified Fab-G8 antibody failed to detect HLA-A1 cells incubated with the MAGE-A1 peptide (see Chames et al., Page 7972, left column, third paragraph), thus could not be used for detecting or killing cells presenting the MHC-peptide complex.

In this regard, Andersen et al. (WO 97/02342) have not provided any further teachings which could advance the use of TCR-like antibodies in human therapy in general and the treatment of virus infections, in particular.

The antibodies described in Andersen et al. (1997) and/or Reiter et al. (1997) are directed against mouse MHC-peptide complexes and not against human MHC-peptide complexes. As the mouse and human MHC molecules exhibit completely different chemical structures, such antibodies (e.g., Fab 13.4) cannot be used for targeting and treating human cells. It is my expert opinion that the antibodies contemplated by Andersen et al. are no more than an expression of a desired result rather than actual conception and reduction to practice.

Thus, it is my opinion that all the antibodies described in the prior art (including the publications of Andersen and Reiter) did not satisfy the long-felt need of generating TCR-like antibodies and killing virus infected human cells.

In addition, I further declare that during the years of 2002-2007 my research lab attempted to identify TCR-like antibodies by screening one library against the NY-ESO-$1_{265-274}$/HLA-A2 complexes. However, these efforts resulted in the lack of lysis of NY-ESO-1 positive tumor cells.

It is further my expert opinion that the claimed invention of the above-referenced patent application in fact satisfies the long felt need for antibodies which are specific to human complexes of antigen-presenting molecules and an antigen derived from a pathogen and therefore can be used for killing virus infected human cells.

Thus, the instant application demonstrates, for the first time, the ability to reproducibly generate antibodies with TCR-like specificity for human complexes composed of antigen presenting molecules and peptides derived from a pathogen. The ability of such antibodies to kill virus infected human cells renders them imperative tools for human therapeutic applications (e.g., for killing cells displaying the MHC-peptide complexes).

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Examiner: Lucas, Zachariah
Group Art Unit: 1648
Attorney Docket: 28429

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

16th July 2007

Vincenzo Cerundolo

Enc.:
CV of Vincenzo Cerundolo

References:

- Tamminene WL., et al., Eur. J. Immunol. 1987, 17:999-1006;
- Rubin B., et al., 1989, Res. Immunol. 140:67-74;
- Chames P., et al., PNAS, 2000, 97:7969-7974;

Curriculum Vitae

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Qualifications

2002	Fellow Royal College of Pathologists (FRCPath)
2002	Fellow of the Academy of Medical Sciences (FMedSci)
2000	Professor of Immunology, University of Oxford
1999	MA, University of Oxford
1995	Membership of Royal College of Pathologists (MRCPath)
1993	General Medical Council Registration (No. 4066655)
1987	PhD in Immunology, University of Padua, Italy
1984	Full registration as medical practitioner in Italy
1984	Medical Degree (cum laude), University of Padua, Italy

Appointments

Since 2004	Associate Director, MRC Human Immunology Unit, Oxford
Since 2001	Professor, University of Oxford
Since 2000	Director, Tumour Immunology CRUK Programme, University of Oxford
Since 1996	Honorary Consultant, Medical Oncology, Oxford Radcliffe Hosptials NHS Trust
1996 – 2001	Medical Research Council Clinical Fellow, Institute of Molecular Medicine, Oxford
1990 – 1995	Medical Research Council Post-Doctoral Fellow, Institute of Molecular Medicine, Oxford
1988 – 1989	Fellow of European Molecular Biology Organization (EMBO), Institute of Molecular Medicine, Oxford
1984 – 1987	Residence in Oncology, Institute of Oncology, University of Padua, Italy
1980 – 1984	Elective in Immunology, Department of Immunology, University of Padua, Italy

Fellowships

2002	Fellow Royal College of Pathologists (FRCPath)

2001	Fellow Academy of Cancer Immunology
1997	Research Fellow, Merton College, Oxford
1996	Clinical Senior Fellow, Medical Research Council
1988	Long-term Fellow, European Molecular Biology Organisation (EMBO)
1984	Fellow, Associazione Italiana per la Ricerca sul Cancro (AIRC)

Prizes

1997	Investigator Award from the Cancer Resarch Institute, USA
1984	Prize Virgilio Chinni for PhD thesis at Padua Medical School, Italy

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Alignment Mouse and Human MHC molecules

```
########################################
# Program:  needle
# Rundate:  Thu Jul 12 12:21:38 2007
# Align_format: srspair
# Report_file: /ebi/extserv/old-work/needle-20070712-12213313046124.output
########################################

#=======================================
#
# Aligned_sequences: 2
# 1: EMBOSS_001 AAA53203 Mouse MHC
# 2: EMBOSS_002 AAA76608 Human MHC
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 381
# Identity:     239/381 (62.7%)
# Similarity:   275/381 (72.2%)
# Gaps:          28/381 ( 7.3%)
# Score: 1218.5
#
#
#=======================================

EMBOSS_001         1    mapcmllllaaalaptqtragphslryfhtavsrpglgkprfisvg     47
                        |||..|:|||:.|||.|||.||.||:|||.|:||||||.|:||||:||
EMBOSS_002         1 mavmaprtlvlllsgalaltqtwagshsmryfftsvsrpgrgeprfiavg     50

EMBOSS_001        48 yvddtqfvrfdsdaenpryeprvrwmeqvepeywerntqiakgneqifrv     97
                     ||||||||||||||.:.|.|||..|:||..||||:..|:..|.:.|..||
EMBOSS_002        51 yvddtqfvrfdsdaasqrmeprapwieqegpeywdgetrkvkahsqthrv    100

EMBOSS_001        98 slrtalryynqsaggshtfqrmygcevgsdwrllrgyeqyaydgcdyial    147
                     .|.|...||||||..||||.||||||:||||||.||||.||||||.|||||
EMBOSS_002       101 dlgtlrgyynqseagshtvqrmygcdvgsdwrflrgyhqyaydgkdyial    150

EMBOSS_001       148 nedlktwtaadmaalitkhkweqagdaerdraylegtcvewlrrylqlgn    197
                     .|||::|||||||||..||||||.|..||:.|||||||||||||||:.|.
EMBOSS_002       151 kedlrswtaadmaaqttkhkweaahvaeqlraylegtcvewlrrylengk    200

EMBOSS_001       198 atlprtdspkahvtrhsrpedkvtlrcwalgfypaditltwqlngeeltq    247
                     .||.|||:||.|:|.|:..:.:.|||||||.||||:||||||.:|||:.||
EMBOSS_002       201 etlqrtdapkthmthhavsdheatlrcwalsfypaeitltwqrdgedqtq    250

EMBOSS_001       248 dmelvetrpagdgtfqkwasvvvplgkeqyytchvyhqglpepltlrwep    297
                     |.||||||||||||||||:||||.|:||.||||||.|:|||:||||||||
EMBOSS_002       251 dtelvetrpagdgtfqkwaavvvpsgqeqrytchvqheglpkpltlrwep    300

EMBOSS_001       298 pps-tvsntviiavlvvlgaaivtgavvafvmkmrrrntaflltggkggd    346
                     ... |:....|||.||:.| |::||||||.||..|:.:..      |||.
EMBOSS_002       301 ssqptipivgiiaglvlfg-avitgavvaavmwrrkssdr------kggs    343

EMBOSS_001       347 yalapgs---qtsdlslpdckvmvhdphsla    374
                     |:.|..|   |.||:||
EMBOSS_002       344 ysqaassdsaqgsdvsl                 360


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Biochem Soc Trans. 2002 Aug;30(4):512-6. Links

Formatting antibody fragments to mediate specific therapeutic functions.

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Monoclonal antibodies are increasingly being used as therapeutic agents in a wide range of indications, including oncology, inflammation and infectious disease. In most cases the basis of the therapeutic function is the high degree of specificity and affinity the antibody-based drug has for its target antigen. However, the mechanism of action (MOA), the way the drug takes advantage of this specificity to mediate a therapeutic effect, varies considerably from drug to drug. Three basic potential categories of MOAs exist: antagonists, agonists and specific delivery mechanisms to target an active function to a particular cell type. The latter functions include selective cell killing, based on Fc-mediated events, recruitment of effector cells, and drug or radioisotope delivery. The majority of these mechanisms are not necessarily optimally mediated by an IgG structure; clearly, in the case of antibody-dependent cellular cytotoxicity or complement-mediated lysis, Fc is required. However, Fab fragments (the fragment comprising one antigen-binding arm of the Y-shaped IgG molecule) can be formatted to mediate most mechanisms and have the advantage that valency and half-life can be controlled to simplify the drug and address only the mechanism required. Moreover, Fab fragments can be produced in microbial expression systems which address manufacturing issues such as scale of supply and cost of goods.

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Searching for MHC-restricted anti-viral antibodies: antibodies recognizing the nucleoprotein of influenza virus dominate the serological response of C57BL/6 mice to syngeneic influenza-infected cells*

An attempt has been made to generate monoclonal antibodies which recognize the same target structures on influenza-infected cells as those seen by cytotoxic T lymphocyte (CTL) receptors. Such antibodies, if they mimicked the T cell receptor specificity, would be expected to be both virus specific and restricted in their binding by the major histocompatibility complex (MHC) antigens. Approximately 200 hybridomas from C57BL/6 (H-2^b) mice primed and boosted with influenza virus (X-31)-infected EL4 (a C57BL/6 T cell lymphoma) were screened for reactivity on infected and uninfected cells of different MHC haplotypes. Of the 10 hybridoma antibodies which were identified as being reactive with X-31-infected EL4, but not uninfected EL4, all reacted equally well with X-31-infected cells of H-2^b, H-2^d and H-2^k haplotypes, indicating a lack of MHC restriction in their recognition of the infected cells. Unexpectedly, 7 of the 10 monoclonal antibodies were found to react specifically with the purified influenza virus nucleoprotein (NP), a predominant viral antigen in CTL recognition of infected cells. Fluorescence-activated flow cytometry confirmed that these antibodies were able to recognize NP serological determinants on the surface of viable, infected cells, but the anti-NP antibodies were unable to block the lytic activity of an NP-specific CTL clone.

1 Introduction

Virus-specific cytotoxic T lymphocytes (CTL) are known to recognize foreign determinants on infected cells in the context of class I major histocompatibility complex (MHC) gene products [1]. Although considerable progress has recently been made in terms of defining the nature of the T cell antigen receptor (reviewed in [2]), much uncertainty remains with respect to the nature of the target structures seen by CTL on the surface of virus-infected cells. The study of CTL target structures and their formation would be greatly facilitated by the availability of monoclonal antibodies (mAB) recognizing structural determinants which either overlap with, or are identical to, those seen by the CTL antigen receptors. Such antibodies would be analogous to soluble T cell receptors, and could provide an important set of immunological tools for defining the nature of CTL target structures.

Wylie et al. [3] previously reported that, using a splenic fragment culture assay, the majority of responding primary B cell clones produced in mice challenged with influenza virus-infected cells secreted antibodies which were MHC restricted in their binding to influenza-infected cells. The majority of these were found to recognize the influenza virus hemagglutinin (HA) in the context of class I MHC determinants. Encouraged by this finding, we undertook to determine if conventional mAb could be generated which would recognize influenza virus determinants in the context of MHC antigens. Spleen cells from mice challenged 2–4 times with viable, influenza A-type virus-infected syngeneic cells were used to produce antibody-secreting hybridoma clones. Of the 10 hybridoma clones specific for influenza virus-infected cells characterized in this study, none produced antibody dependent on the haplotype of the infected target cell for binding. Surprisingly, 7 of the 10 mAb were found to be specific for the influenza nucleoprotein (NP), the component previously identified as being immunodominant with respect to influenza virus-specific CTL [4]. Although the NP-specific mAb antibodies were able to detect NP serological determinants on the surface of viable infected cells, they were unable to block lysis of influenza-infected cells by an NP-specific CTL clone.

2 Materials and methods

2.1 Viruses

All influenza viruses were grown from seed stock generously provided by J. J. Skehel (WHO Influenza Reference Center, Mill Hill, GB). Virus was grown at 34 °C in the allantoic cavity of 10-day-old embryonated chicken eggs. Infectious allantoic fluid was isolated, titred for hemagglutination activity against chicken red blood cells and stored at −70 °C. The following strains of influenza virus were used: natural isolates; A/PR/8/34(H1N1), A/Hong Kong/1/68(H3N2), A/Texas/1/77(H3N2) and B/Hong Kong/8/73 and recombinant viruses isolated from mixed infections with A/PR/8/34 and post-1968 H3N2 viruses; X-31, X-47, E61-24-H16 and E61-13-H17. The genotypes of the recombinants are shown in Table 1 as previously described [5, 6].

[I 5929]

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Abbreviations: MHC: Major histocompatibility complex NP: Nucleoprotein HA: Hemagglutinin NA: Neuraminidase CTL: Cytotoxic T lymphocyte(s) PBS: Phosphate-buffered saline ELISA: Enzyme-linked immunosorbent assay RIA: Radioimmunoassay HAT: Hypoxanthine aminopterin thymidine PEG: Polyethylene glycol mAb: Monoclonal antibody(ies) BSA: Bovine serum albumin

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Table 1. Genotypes of recombinant viruses[a)]

Virus	PB1	PA	PB2	HA	NA	NP	M	NS
X-31	x	x	x	o	o	x	x	x
X-47	o	x	x	o	o	o	x	x
H17	x	x	x	o	o	o	x	x
H16	o	x	x	o	o	x	x	o

a) x denotes a gene derived from A/PR/8/34; o denotes a gene derived from post-1968 H3N2 viruses; PB1, PA, PB2, viral polymerases; M, matrix protein; NS, nonstructural proteins.

2.2 Cell lines

The following cell lines, obtained from the American Type Culture Collection (Rockville, MD), were used: EL4 (H-2^b, T cell lymphoma) [7], P815 (H-2^d, mastocytoma) [8], BW5147.G.1.4. (H-2^k, T cell lymphoma) [7] and Sp2/0 H-2^d, H chain$^-$, L chain$^-$ myeloma) [9]. Cell lines were maintained in culture in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM penicillin/streptomycin, 2 mM L-glutamine and, in the case of Sp2/0, 2 mM sodium pyruvate. All cultures were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.3 mAb

The antibody-producing lines HB-11 (20-8-4S, anti-K^bD^b) [10], HB-20 (3-83-P, anti-H-$2K^kD^k$) [11] and HB-65 (H16-L-10-HRS, anti-FluA NP) [12] were obtained from ATCC, and Y3 (anti-H-$2K^b$) was kindly provided by C. Janeway. The corresponding mAb were utilized as culture supernatants. HC-3, a monoclonal anti-HA (H3) ascites fluid, was generously supplied by J. J.Skehel. Anti-phosphorylcholine (anti-PC) antibody, PC28/3-7, was obtained from M. Shulman.

2.4 Influenza virus infection of murine tumor cells

Tumor cells were harvested and washed twice with serum-free medium before being resuspended with 200–500 µl of infectious allantoic fluid (500 hemagglutinating units, HAU/1 × 10^7 cells) and an equal volume of serum-free medium. Incubation was for 1 h (37 °C, 7% CO_2) with agitation every 15 min. Cells were then washed twice with RPMI 1640 containing 10% FBS and subsequently placed in culture flasks at approximately 1 × 10^6 cell/ml in RPMI 1640/2 mM penicillin-streptomycin/10% FBS/2 mM L-glutamine. Flasks were returned to the incubator for 5–12 h to allow for expression of viral proteins after which cells were harvested for use.

2.5 Immunization of mice with syngeneic infected tumor cells

X-31-infected EL4 tumor cells were washed and resuspended in phosphate-buffered saline (PBS) at 1 × 10^7 cells/ml. C57BL/6J (H-2^b) mice, 20–36 weeks old, obtained from Jackson Laboratories (Bar Harbor, ME), were challenged with 1 × 10^7 infected cells by i.p. injection. Mice were re-challenged once or twice in the same way with 3–4 weeks spacing between injections. At 3–8 weeks after the final immunization, the mice were given an i.p. or i.v. boost identical to the previous immunization(s) and the immune spleen cells were used 3–4 days later for fusion.

2.6 Fusion of immune spleen cells with Sp2/0

The fusion protocol was adapted from the procedure suggested by De St. Groth and Scheidegger [13]. Antibiotics (2 mM penicillin/streptomycin) were included in all media and buffers used. In general, spleens from 2 immune mice were pooled. The fusion parter, Sp2/0, was harvested from culture at no greater than 3 × 10^5–4 × 10^5 cells/ml, washed and resuspended in RPMI 1640/10 mM HEPES (HEPES 1640). Equal numbers of immune spleen cells and Sp2/0 (4 × 10^7–5 × 10^7 each) were combined, washed once with HEPES 1640, and fused with 1.0 ml of polyethylene glycol (PEG 4000, Merck, Darmstadt, FRG, 50% in HEPES 1640) added dropwise over 1 min. After agitation at 37 °C for 90 s the fusion was stopped by addition of 20 ml HEPES 1640. An equal volume of RPMI 1640/10% FBS was added and the cells were left to stand at room temperature for 5 min before suspending and pelleting. Cells were then resuspended to 5 × 10^6/ml in RPMI 1640/10% FBS/HAT/L-glutamine/sodium pyruvate (HAT medium). Cells (2.5 × 10^5) were then seeded into each well of 24-well plates previously prepared with a feeder layer of C57BL/6J spleen cells. The plates were placed in a humidified incubator with 5% CO_2.

On day 7 after the fusion, 1 ml of the HAT medium was added to each well, and on days 10, 12 and 14, wells were fed with fresh HAT medium. Between days 14 and 21 wells were fed with HT medium (*i.e.* HAT medium lacking aminopterin) and thereafter maintained on RPMI 1640 containing 10% FBS, L-glutamine, sodium pyruvate and antibiotics. Supernatants were collected for testing when wells were half-confluent and then stored at −20 °C. Representative positive wells were later selected for cloning by limiting dilution. In some instances, clones were expanded for larger scale antibody production. Antibodies were purified by protein A-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography (according to Ey et al. [14]) and concentrated to > 1 mg/ml by pressure ultrafiltration (Amicon, Lexington, MA). Purified mAb were stored at 4 °C in the presence of 10 mM sodium azide.

2.7 Preparation of antigen plates for enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA)

Three types of antigen targets were prepared using flexible, round-bottom, 96-well polyvinyl chloride microtiter plates (Linbro, New Haven, CT).

(a) Cellular targets. These were prepared using modifications of methods described in the literature [15, 16]. Infected or uninfected tumor cells were harvested from culture, washed three times in PBS and resupended at 1 × 10^6 cells/ml. A volume of 100 µl was dispensed into each well of target plates which had been pre-treated with poly-L-lysine (0.05 mg/ml: 50 µl/well) for 45 min at room temperature. The plates were then centrifuged at 400 × g for 7 min at room temperature (RT) and 100 µl of 0.2% glutaraldehyde was added for 5 min at RT. The plates were then flicked and washed three times with PBS. Nonspecific binding sites were blocked by filling the wells with 0.5% bovine serum albumin (BSA)/0.02% sodium

azide/PBS (blocking buffer) and incubating for 1 h at RT. The plates were either used immediately or stored at 4 °C for up to several months without removal of the blocking buffer.
(b) Whole virus targets. Infectious allantoic fluid was diluted to 20 HAU/25 µl with PBS; 25 µl (20 HAU) of the viral solution was dispensed per well and the plates were left to air-dry overnight. The plates were then incubated with blocking buffer and stored as for the cellular targets. As a control, plates were also prepared using uninfected (normal) allantoic fluid.
(c) Purified viral protein targets. Plates were prepared with 0.4 µg/well of NP purified from A/X-31 [17]. These wells were incubated with blocking buffer for 1 h before use. The bromelain fragment of the HA (BHA) from X-31 was kindly supplied by J. J. Skehel. Purified neuraminidase (NA) from A/Jap/305/57 (H2N2) was obtained commercially (Calbiochem, Los Angeles, CA). The BHA and NA were diluted in PBS to concentrations of 5–625 µg/ml and 50 µl was dispensed per well. After incubating for 1 h at RT, plates were flicked, blocked as above and stored as for the cellular targets.

2.8 ELISA and RIA

Each mAb of interest was diluted to an appropriate concentration with blocking buffer. The assay wells were flicked to remove the blocking buffer and blotted dry. Fifty µl of sample antibody was added per assay well and incubated for 1–2 h at RT. The antibody was then flicked off and the wells were washed three times with PBS. If performing an ELISA, 100 µl of urease-conjugated rabbit anti-mouse Ig [µ and γ chain-specific antibody (Allelix, Toronto, Canada) 1 : 500 dilution] was added to the wells and allowed to incubate for 45 min at 34 °C. After flicking off the antibody-enzyme conjugate, the wells were washed three times in PBS followed by three washes with deionized water. One hundred µl of urease substrate solution (Allelix) was then added to each well. Color development after 15–20 min was determined by absorbance at 595 nm. In performing a RIA, instead of adding the urease-conjugate as the second antibody, 50 µl of an ^{125}I-labeled protein A (Amersham Int., Bucks, GB), prod. n. IM.112) solution containing 50 000–60 000 cpm/50 µl was added to each well and incubated for 1–2 h at RT. Unbound ^{125}I-protein A was removed by washing three times with PBS. The wells were dried, cut out and counted in a γ-counter.

2.9 Flow cytometry of influenza-virus infected cells

X-31-infected EL4 tumor cells were harvested and washed two times in PBS. Cells were distributed into 10 ml round-bottom centrifuge tubes at 1.2×10^6 cells/tube and washed once more in RPMI 1640/0.1% BSA/0.1% sodium azide (binding buffer), spinning at 400 × g for 8 min at RT. The supernatant was removed and the pellet gently resuspended in 100 µl each of ice-cold binding buffer and sample antibody. The tubes were incubated on ice for 1 h, with agitation every 10 min. After washing twice at 4 °C with binding buffer, the pellet was resuspended with 100 µl of a 1 : 20 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibody (Cappel Laboratories, Cochranville, PA). The tubes were incubated on ice for 30 min, agitating every 10 min. The cells were then washed once in binding buffer, once in PBS, and resuspended in 1 ml of PBS. Immediately before analysis on the EPIC V flow cytometer (Coulter, Harpenden, GB), each sample was mixed with 1/10 volume of propidium iodide (1 mg/ml). Propidium iodide was added to stain the DNA of nonviable cells, allowing their exclusion from analysis [18]. For purposes of comparison, mean peak fluorescence intensities were converted from logarithmic to relative fluorescence intensities (RFI) by a standard arithmetic relationship.

2.10 Generation and assay of an anti-influenza CTL

The A-type influenza-specific CTL clone, B4, was established from X-31-infected C57BL/6 mice using the protocol described by Townsend and Skehel [19]. The specificity of the clone was established using a standard ^{51}Cr-release assay for cell-mediated cytotoxicity with different target cells infected with a series of recombinant influenza virus strains [20]. When the mAb were tested for their ability to inhibit the lytic activity of B4, target cells were incubated for 30 min with a 1/2 dilution of the mAb-containing culture supernatants before assay, and a 1/4 dilution of the culture supernatant was maintained throughout the 4-h assay period.

3 Results

3.1 Identification of hybridomas reactive with the infected EL4 cell surface

Hybridoma culture wells containing immunogen-specific antibody were screened by ELISA for their ability to react with X-31-infected EL4 (EL4 + X-31), and failure to react with uninfected EL4 cells. Of 189 clones screened, 10 were identified with this specificity. Under the limiting dilution conditions of the initial HAT selection step, the percentage of wells positive for growth in each fusion was less than 50%, while the percentage of wells reactive with infected but not uninfected EL4 was less than 3%. These frequencies strongly suggest that the virus-specific antibodies from any of the 10 positive wells are very likely to be monoclonal. This conclusion was further supported when 4 of the 10 positive wells were later subcloned and found to retain their original specificity (data not shown).

3.2 mAb reactivity with infected cells of different haplotypes

To test for involvement of class I MHC antigens in the formation of infection-specific serological determinants, the antibodies were screened by RIA for differential reactivity on X-31-infected tumor cells of the b, d and k haplotypes. If any of the hybridoma antibodies were specific for influenza antigens in the context of self-MHC antigens, they would be expected to react only with X-31-infected target cells of the b haplotype, since the immunogen and responding host were of this haplotype. Using a comparative binding assay, possible differential reactivity of a given hybridoma with infected cells of different haplotypes could be masked by the presence in the same fusion well of a hybridoma strictly specific for an influenza antigen, since antibody of such specificity would bind to X-31-infected targets of all haplotypes. It was for this reason that we incorporated a cloning step into the initial hybridoma selection procedure after fusion.

Using the control antibodies HC-3 and HB-65 specific for HA and NP, respectively, it was found that, under conditions of antibody saturation, there was an inherent inequality among the different cell types in terms of their expression of HA and

NP. This made it impossible to compare directly the binding data for the cells of different haplotypes. P815 (H-2^d) showed low levels of detectable viral proteins relative to EL4 (H-2^b) and BW5147 (H-2^k). The surface HA expression with another k haplotype T cell lymphoma, RDM-4 was also lower than that with EL4 or BW5147, indicating that the reduced level of expression seen with P815 is not unique to the d haplotype (data not shown). The reason for this variation among cell types is not known, but might be due to differences in their inherent susceptibility to infection or different rates of viral protein synthesis. In order to permit a comparison of antibody binding on cells of different haplotype, the control levels of anti-HA and anti-NP binding at saturation were taken as an indicator of the availability of viral determinants on the various infected cell types. Assay results were then normalized by expressing the binding of each sample antibody as a fraction of the binding of either the anti-HA or anti-NP control. The results, both absolute and relative, are summarized in Table 2. As an example of how important this normalization turned out to be, the absolute results for EX9 indicate that binding on infected EL4 and BW5147 cells was roughly 5–6 times greater than binding on infected P815, which might be interpreted as a true difference in affinity of the antibody for the different targets. However, once the results are normalized to account for differences in the amounts of viral determinants available, the antibody is seen to have little preference for one target haplotype over another. Of the 10 antibodies tested, none showed significant differences in binding as a function of infected-cell haplotype, indicating a lack of involvement of the class I molecules in formation of the serological determinants recognizing by the antibodies.

Table 2. mAb reactivity with infected cells of different haplotype

mAb		Infected cell target (cpm bound)[a]		
		EL4 + X-31 (H-2^b)	P815 + X-31 (H-2^d)	BW5147 + X-31 (H-2^k)
HC-3 (anti-HA)		19256	3432	30792
HB-65 (anti-NP)		11107	1568	15307
EX1		11107	1730	8591
	ratio to HA	0.58	0.50	0.28
	ratio to NP	1.00	1.10	0.57
EX2		14468	2234	7281
	ratio to HA	0.75	0.65	0.24
	ratio to NP	1.30	1.42	0.48
EX3		8544	1588	4128
	ratio to HA	0.44	0.46	0.13
	ratio to NP	0.77	1.01	0.27
EX4		2128	255	170
	ratio to HA	0.11	0.07	0.005
	ratio to NP	0.19	0.16	0.011
EX5		6783	3312	45938
	ratio to HA	0.35	0.96	0.80
	ratio to NP	0.61	2.11	1.85
EX6		5574	3915	20512
	ratio to HA	0.29	1.14	0.04
	ratio to NP	0.50	2.50	0.38
EX7		5240	548	3436
	ratio to HA	0.18	0.16	0.17
	ratio to NP	0.40	0.35	0.40
EX8		9652	2254	9503
	ratio to HA	0.50	0.66	0.48
	ratio to NP	1.11	1.44	1.10
EX9		7691	1414	9187
	ratio to HA	0.40	0.41	0.46
	ratio to NP	0.89	0.90	1.07
EX10		8057	2542	9425
	ratio to HA	0.42	0.74	0.48
	ratio to NP	0.93	1.62	1.10

a) Values represent the mean of duplicates unless otherwise noted.
b) Uninfected target cells had background levels of 200–400 cpm; see Table 3.
c) Single point assay.

3.3 mAb reactivity with different influenza-virus-infected targets

The lack of MHC restriction of the 10 antibodies suggested that they were likely specific for the influenza antigens alone. On the basis of previously reported B cell responses to both influenza virus and influenza-infected cells [3, 21, 22], we expected that the viral glycoproteins would be the dominant antigens recognized by the infected cell-specific antibodies. In order to test for such glycoprotein specificity, the reactivity of the antibodies with X-31 and PR8-infected EL4 cells was compared. The immunizing recombinant strain, X-31, derives its HA and NA genes from A/HK/1/68(H3N2) and all other genes from A/PR/8/34(H1N1) as shown in Table 1. Since H3 and N2 are serologically non-cross-reactive with H1 and N1, respectively [23], an antibody-recognizing glycoprotein determinants should react with X-31-, but not PR8-infected EL4. On the other hand, if nonglycosylated viral proteins were involved in determinant formation, the antibody specific for these epitopes would react with both X-31- and PR8-infected EL4. Representative RIA results of the reactivity of the antibodies against both X-31- and PR8-infected EL4 are shown in Table 3. Surprisingly, it was found that 8 of the 10 antibodies were reactive with both infected targets, suggesting that the majority of the antibodies recognized determinants formed by the nonglycosylated viral components, and not the glycoproteins. Although it is possible that there might exist serological

Table 3. mAb reactivity with different influenza virus-infected targets

mAb	Infected cell target (cpm bound)[a]		
	EL4	EL4 + X-31 (H3N2)	EL4 + PR8 (H1N1)
HC-3 (anti-H3)	254	23697	207
HB-65 (anti-NP)	198	15357	5901
HB-20 (anti-H-2D^d)	788	522	352
EX1	429	15097	6791
EX2	446	14388	7281
EX3	242	18352	8544
EX4	202	2260	2128
EX5	125	9284	170
EX6	118	9432	69
EX7	436	14468	6783
EX8	199	9878	5574
EX9	182	12384	5240
EX10	412	9154	4244

a) All values represent the mean of duplicates.
b) In this and subsequent tables responses considered significantly above background levels are underlined.

(EX3, 7, 8 and 9) cross-reacted on all four A-type strains tested, although the binding of EX8 and EX9 to HK was relatively weak. These findings indicate that this group of anti-NP monoclonals recognize a serological determinant common to both the PR8 and HK-derived NP.

The NP of influenza virus has recently been divided into two subgroups based on the ability of influenza-stimulated CTL to differentiate between the NP from these groups. One subgroup consists of the isolates from 1934–1943 (A/PR/8/34-related) while those from 1946–1979 (A/HK/8/68-related) form the second subgroup [20]. It was thus interesting that EX1 and EX2 could discriminate between targets in the same way expected for a subtype-specific CTL, reacting only with the pre-1943 NP of PR8 and not with the HK-related NP of the post-1943 isolates, HK and Texas.

3.6 Serological detection of NP determinants on viable, influenza-infected EL4 cells

It was necessary to consider the possibility that the NP detected in the RIA with fixed target cells was not expressed on the cell surface, but rather only available in the cytoplasm of infected cells rendered permeable by the glutaraldehyde fixation. To determine whether or not NP determinants were present on the surface of infected cells, affinity-purified anti-NP mAb were used to assess reactivity with intact X-31-infected EL4 cells using a fluorescence-activated cell sorter. Fig. 1 depicts the results of this experiment. Panel A shows that a low level of background fluorescence (5–6% positive cells) was obtained with a control antibody. Under conditions of antibody saturation, the subtype-specific anti-NP antibody, EX2 (Fig. 1B), stained 90–95% of the infected cell population, whereas the A-type cross-reactive anti-NP antibody, EX8, stained about 50% of the cells. Interestingly, HB-65, the established positive anti-NP control, which is A-type cross-reactive like EX8, had a fluorescence profile (Fig. 1F) almost identical to that of EX8 (Fig. 1C). Although the reason for the difference in distribution of cell surface NP determinants detected using different anti-NP antibodies is not known, the findings suggest a correlation between determinant distribution and subtype specificity. The anti-HA antibody, EX6 (Fig. 1E), and an anti-class I histocompatibility antigen control antibody, HB-11 (Fig. 1D), labeled 99% and 100% of the infected cells, respectively. Conversion of the mean fluorescence intensities from the logarithmic scale of Fig. 1 to relative fluorescence intensities indicated that the amount of cell surface NP detected with either of the anti-NP antibodies was approximately 10% of HA on the cell surface and 17% the amount of class I determinants detected. The broad peak observed with the anti-HA antibody (Fig. 1E) reflected considerable heterogeneity in the number of HA molecules expressed on different infected cells. In contrast, the population was relatively homogeneous in terms of cell surface expression of NP and class I determinants, as indicated by the relatively narrow distribution of fluorescent cells labeled by these antibodies.

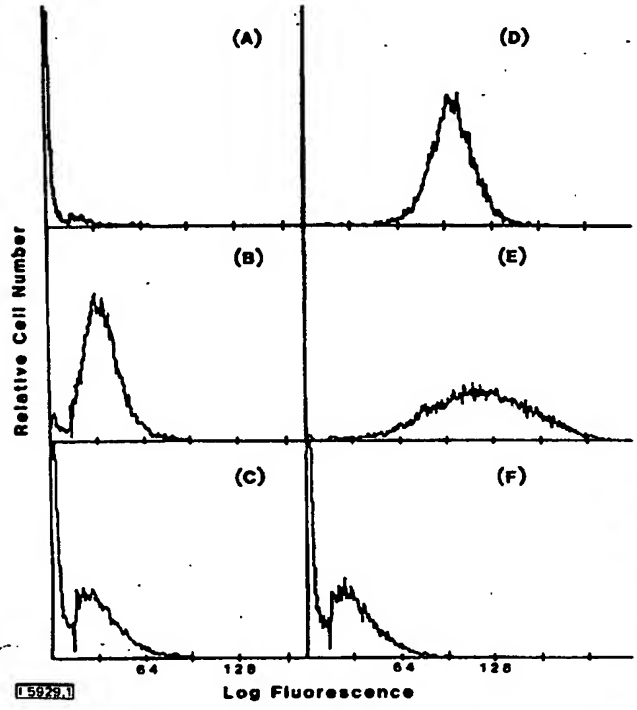

Figure 1. Fluorescence analysis of cell surface antigens on A/X-31-infected EL4 tumor cells. Distribution of surface antigens detected with (A) anti-phosphorylcholine (nonspecific binding control); (B) EX2 (subtype-specific anti-NP); (C) EX8 (A-type cross-reactive anti-NP); (D) HB-11 (anti-H-2K^{b}D^b); (E) EX6 (anti-HA); (F) HB-65 (anti-NP control).

3.7 Can the NP-specific antibodies block an influenza-specific CTL clone which recognizes the A/PR/8/34 NP?

The reactivity profile of B4 (a C57BL/6-derived influenza A-type specific CTL clone) is depicted in Table 7. B4 can recognize and lyse PR8-infected peritoneal exudate cells from B10.HTG (K^{d}D^d) mice, but not from B10.A(5R) (K^{b}D^d) mice, indicating that the clone is restricted in its recognition by H-2D^b. A/X-31 is a recombinant influenza virus which possesses the HA and NA genes of the 1968 A/HK strain in association with the internal structural genes of the 1934 PR8 strain. The observation that B4 was able to lyse PR8- and X-31-infected cells, but not HK-infected cells, indicated its specific-

Table 7. Specificity of anti-influenza CTL clone C57.B1.6 B4

Cell			% Lysis
			24
			2
			−3
			66
			0
			67
			2
			1
			38
			34
			34

a) Target cells from these mouse strains were thioglycolate-induced peritoneal exudate cells.

ity for one of the internal virus components. Further analysis using target cells infected with additional recombinant viruses (Tables 1 and 7) mapped the internal component specificity to the NP gene.

Having established the NP specificity of the B4 clone, attempts were made to inhibit its CTL activity with two representative NP-specific mAb from those generated to X-31-infected EL4 cells. The results in Table 7 indicate that even under conditions where antibody binding to the target cells is expected to be in saturation, neither the A-type cross-reactive (EX3) nor the subtype-specific (EX2) anti-NP mAb were able to inhibit CTL activity significantly.

4 Discussion

The aim of this study was to determine whether or not hybridoma-derived mAb could be generated which would react with the cell surface determinants recognized by anti-influenza CTL clones. These antibodies, like antigen receptors on the CTL, would be expected to be both MHC restricted and virus specific [1]. The possibility that MHC-restricted antibodies could be generated is supported by a number of recent reports in the literature [3, 24–26]. In particular, the study of the B cell responses to immunization with syngeneic, influenza-infected tumor cells by Wylie and Klinman [21] indicated that approximately two thirds of the anti-viral antibodies recovered in a splenic fragment assay of a primary response were specific for determinants present on infected cells but not on purified virus. Further analysis [3] showed that 60% of these antibodies displayed a CTL-like MHC restriction, binding only to infected cells bearing class I antigens of the immunizing haplotype. Similar results have recently been reported by Froscher and Klinman [26] for mAb responses to SV-40 virus-transformed cells. We found that none of the 10 infected-cell-specific mAb generated in C57BL/6 mice after multiple injections of syngeneic, influenza-infected EL4 cells were restricted by the MHC in their recognition of the viral antigens. Our antibodies bound equally well to infected cells which were either allogeneic or syngeneic to the immunogen. In this context, it is important to note that we found a significant quantitative difference in the cell surface expression of influenza virus components among the different cell types used in this assay. If the binding results had not been normalized to the control values, a correlation between target cell haplotype and the degree of antibody binding might have been mistakenly assigned (see Table 2).

In addition to the lack of any apparent MHC restriction of antibody binding, each of the antibodies tested was able to react with the virus itself, and also with purified viral components, precluding any necessary involvement of cell surface components in antigen recognition by these antibodies. Although the analysis of 10 clones is by no means extensive, these results do suggest that the cell-surface target structures recognized by influenza-specific CTL may not be as serologically potent as previously thought. The contrasting high frequency of MHC-restricted antibodies observed by Wylie et al. [3] may be related to (a) differences in the immunization protocols which were used (*e.g.* primary *vs.* multiple boost responses), (b) the methods used to analyze monoclonal responses, or (c) the different A-type influenza viruses used to prepare the immunogens. It is clearly important to establish whether or not the majority of anti-viral antibodies are MHC restricted, and the situation will only be clarified by a more complete analysis of the unique serological determinants created on the surface of influenza-infected cells.

Another result of this study was the unexpected finding that the majority of B cells responding to immunization with influenza-infected cells were specific for viral NP determinants. Although previous reports have indicated the presence of NP on the surface of influenza-infected cells [12, 27], only recently has its involvement in CTL recognition been established [4]. In contrast with the high frequency of anti-NP antibodies observed in this study with infected cell immunogens, the predominant antibody response to immunization with virus alone is directed towards HA and NA determinants [22]. Our results suggest that in a natural infection, cell surface NP determinants may become more important in the serological response after a period of host cell infection and expression of the viral genome. This notion is supported by the work of Gerhard et al. [22] which showed that, in their preparation of mAb from mice challenged with viable virus, a long wait between a final boost and subsequent fusion resulted in a relative rise in the frequency of mAb specific for internal components, with a corresponding decrease in frequency of HA- and NA-specific antibodies.

Although recent studies have shown that the majority of influenza-specific CTL recognize the viral NP [4], the mechanism by which the NP participates in the formation of a class I-restricted target structure seen by the CTL is still unknown. Because the intact NP molecule lacks a signal sequence and is not glycosylated, it is not expected to be found on the surface of viable, infected cells, making its involvement in the CTL recognition event somewhat of a mystery. Thus, it was of interest to determine the nature of the NP determinants recognized by the anti-NP antibodies generated in response to the challenge with infected cells. Binding studies using different viral targets showed that while 4 of the anti-NP mAb bound to the NP of both the PR8 and HK-related subtypes, only two reacted exclusively with the PR8 NP. A representative of each type was subcloned, purified and used in a flow cytometry analysis of infected EL4 cells (Fig. 1). The results confirmed that both subtype-specific and cross-reactive NP serological determinants were detectable on the surface of intact infected cells. Relative fluorescence intensities showed that the determinants on positively staining cells were present at 10% and 17% the amount of HA and class I MHC determinants, respectively. Since both class I molecules [28] and HA [29] have been estimated to be expressed on the cell surface at about 10^5 copies per cell, these results suggest a minimum of about 10^4 copies of NP, or serologically intact fragments derived from NP, on the infected cell surface. This estimate corresponds closely to that of 3×10^4 molecules per infected cell previously reported by Yewdell et al. [12] for influenza-infected P815 cells using other methods.

Given that the flow cytometry analysis indicated ten times more HA than NP on the surface of infected EL4 cells, it is even more noteworthy that the response to this immunogen was dominanted by anti-NP specificities. The results strongly suggest that NP determinants may be more immunogenic than the virus glycoproteins when expressed on the infected cell surface. It is known that passive immunization with anti-NP antibodies does not protect mice from influenza infection [30]. However, serum antibodies specific for viral antigens are able

to lyse virus-infected cells through a complement-mediated mechanism [31], so the NP-specific antibodies may play a role in promoting recovery from an established virus infection.

Surprisingly, under conditions of antibody saturation the subtype-specific antibody, EX2, detected NP determinants on about 90% of the cells. In contrast, both our cross-reactive anti-NP antibody, EX8, and a previously isolated cross-reactive anti-NP antibody (HB65) reacted with only 50% of the same infected cell population. These results suggest that various NP determinants may have different capacities to reach the infected cell surface, or do so using different pathways. This finding may be related to the recent observation by Townsend et al. [32] that in cells transfected with the NP gene, the amino- and carboxy-terminal portions of the protein appear capable of independently reaching the point of CTL recognition.

In an attempt to determine whether or not the serological detection of NP on the surface of infected cells was in any way related to the recognition of NP determinants by CTL, we tested both the subtype-specific (EX2) and the A-type cross-reactive (EX3) anti-NP mAb for their ability to inhibit infected cell lysis mediated by the NP-specific CTL clone B4. Inasmuch as the CTL clone B4 had been shown to be specific for the PR8 NP, it was expected that the mAb EX2, which is also specific for the PR8 NP, would be the most likely to inhibit recognition and lysis. However, neither EX2 nor EX3 were effective as inhibitors of the B4 clone recognition of X-31-infected EL4 cells. Certainly the inability of anti-viral antibodies (either polyclonal or monoclonal) to block lysis by virus-specific CTL is not a unique result, as the vast majority of such attempts have failed to yield positive results [4, 33]. Possible explanations for a negative result include a spatial separation of the serological and T cell-reactive sites on the NP molecule,such that antibody binding to the NP expressed on the cell surface would be unable to interfere sterically with the MHC-restricted recognition of NP by the CTL antigen receptor. Alternatively, the serologically detected NP determinants may be on separate fragments of NP from those which are detected by the T cell receptor. Quite recently, Townsend et al. [34] reported that synthetic peptides corresponding to a defined region of the NP amino acid sequence have the ability to form the MHC-restricted target structures recognized by influenza NP-specific CTL clones. Previously these same workers reported that cells transfected with truncated NP genes could also be recognized by NP-specific CTL clones under conditions where NP serological determinants were not appreciably detectable on the surface of the transfected target cells [32]. These findings, and our inability to inhibit an NP-specific CTL clone with either the subtype-specific or cross-reactive mAb, support the notion that the serologically detectable and T cell-reactive forms of the NP molecule represent distinct entities on the infected cell surface. Determining whether the serologically detectable NP is biosynthesized by the cell on which it appears, or is acquired from neighboring infected cells by a process of adsorption, may help clarify this uncertainty. We are presently making use of the anti-NP mAb to address this question.

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RECOGNITION OF INSULIN ON MHC-CLASS-II-EXPRESSING L929 CELLS BY ANTIBODY AND T CELLS

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SUMMARY

In the present experiments, we attempted to obtain evidence that T-cell receptors (Tcr) and immunoglobulins can react against the same antigen-peptide/MHC class II complexes on antigen-presenting cells (APC). Use was made of monoclonal APC, *i.e.* I-A^k α/β gene-transfected L929 fibroblasts, monoclonal anti-insulin antibodies and selected insulin-specific T-cell lines. Evidence for similarities in antibody and T-cell recognition of insulin presented by I-A^k α/β gene-transfected L-cell fibroblasts was not obtained after serious attempts. Furthermore, we found no evidence for synthesis of antibodies specific for insulin-peptide/MHC class II complexes.

KEY-WORDS: Insulin, MHC; APC, Peptide, Tcr.

INTRODUCTION

The central event in induction of immune responses is the activation of helper T cells by antigenic peptides associated with MHC class II molecules on APC [1, 2]. In contrast to this are antibody molecules that recognize soluble antigens or their fragments. However, since Tcr and antibody are of the same family and of rather similar overall structure [8], it is difficult to

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understand why Tcr should react differently from antibody. One major difference in the two recognition systems is that Tcr molecules are cell-bound, whereas antibodies circulate in soluble form. Thus, the reactivity of Tcr may be guided by other T-cell membrane molecules such as CD4 molecules [7], rather than fundamentally differing from the reactivity of antibody molecules. In the present study, we attempted to search for similarities in antibody and T-cell recognition of insulin presented by I-A^k α/β gene-transfected L-cell fibroblasts.

MATERIALS AND METHODS

Animals.

Inbred mice were from the breeding facility of Statens Seruminstitut; they were C_3H/Sscl, B10.BR, B10.A and B10.A (4R), used at the age of 6-12 weeks and immunized with insulin in complete Freund's adjuvant (50 μg insulin/mouse). Thirty min before injection, the mice received 0.2 ml of 500 mg dextrose per ml to prevent hypoglycaemia.

Antigens and antibodies.

Crystallized insulin from pork or sheep (PIns or SIns) were obtained from Novo Biolabs. Eighteen mAb against BIns (bovine) or HIns (human) were obtained from Novo Biolabs [21]; mAb against MHC molecules were provided by Dr M. Pierres [13] or Dr A.F. Williams [11]. Horseradish peroxidase (HRP) coupled rabbit antimouse immunoglobulin antibodies were bought from Dakopatts (Copenhagen, Denmark).

Cells.

MHC-class-II gene-transfected L cells used in the present study were CA 14.11.14 (I-A^k) and CA 36.1.3 (I-E^k). They were cultured in HAT-containing 5 % FCS (foetal calf serum)/RPMI-1640 medium supplemented as before [10]. L929 (H-2^k) or IT.22 (H-2^q) fibroblasts were grown in medium without HAT. Ins-specific T-cell lines were produced as described previously [17] except that the responding $CD4^+/CD8^-$ T cells were purified from lymph node/spleen cell mixtures [18].

ELISA.

The assay used has been described previously [15]. Insulins were coated to the microtitre plates at 10 μg/ml and the different fibroblasts were seeded at 10^5-2×10^5 cells/well. The only change in procedure was the use of either HRP-labelled second antibody or biotin-labelled mAb followed by HRP-labelled avidin.

APC = antigen-presenting cell.
FCS = foetal calf serum.
HIns = human insulin.
HRP = horseradish peroxidase.
mAb = monoclonal antibody.
MHC = major histocompatibility complex.
PIns = pork insulin.
SIns = sheep insulin.
Tcr = T-cell receptor.

RESULTS

General protocol.

The following protocol was adapted for the experiments to be described: CA14 cells were incubated for 2 h with 500 μg Ins/ml of RPMI-1640 (R) at 37°C. Then, 10 % FCS/R was added to give a cell concentration of 10^6/ml and 100 μl were seeded in wells of microtitre plates. After overnight incubation at 37°C, the plates were washed with 1 % FCS/R, incubated for 1 h at 37°C and washed again. Then anti-insulin mAb were added and incubated for 3 h at 37°C followed by wash and incubation at room temperature with HRP-labelled rabbit anti-mouse Ig antibodies for 1 h. The enzyme reaction was performed at room temperature (15 min) and the OD^{492} was read in a spectrophotometer. Using TNP-PIns-coated ELISA plates and a purified anti-DNP mAb in the ELISA with HRP-labelled rabbit anti-mouse Ig antibodies, we could estimate that the following OD_{492} values corresponded to approximately the indicated number of Ab molecules per cell: 0.250 = 2,000/cell, 0.500 = 10,000/cell, 1,000 = 50,000/cell and 2,000 = 250,000/cell.

Detection of insulin on CA14 cells.

PIns and SIns were incubated with CA14 cells and insulin binding was detected by our anti-Ins mAb. A representative experiment is given in table I. The basic finding was that certain mAb reacted with one but not with the other insulin molecule when these were bound to the CA14 cells: OxI-004 reacted with SIns but not with PIns; HUI-018 reacted with PIns but not with SIns. OxI-002 reacted with both insulins and OxI-001, -003 and HUI-004 did not react with any of the insulins on CA14 cells (not shown).

TABLE I. — **Insulin-pulsed fibroblasts of different type react in a similar manner with anti-Ins mAb.**

Target cells	Pulsed with	OD_{492} values in cellular ELISA Anti-Ins mAb: Medium	OxI-004	HUI-018	OxI-002	Anti-MHC
CA 14	SIns	0.057	*1.331*	0.206	*1.322*	*1.919* (1)
	PIns	0.043	0.198	*1.708*	*1.023*	*1.823*
CA 36	SIns	0.083	*1.322*	0.173	*1.008*	*1.738* (2)
	PIns	0.079	0.168	*0.989*	*1.090*	*1.821*
L929	SIns	0.113	*0.867*	0.073	*1.390*	*2.000* (3)
	PIns	0.089	0.098	*1.029*	*1.239*	*2.000*
IT.22	SIns	0.097	*1.486*	0.211	*1.078*	*1.321* (4)
	PIns	0.045	0.270	*1.173*	*1.196*	*1.557*

Target cells were pulsed with SIns or PIns for 18 h at 37°C before ELISA. Anti-MHC antibodies: (1) Ox6 = anti-I-A^k, (2) 41.A = anti-I-E^k, (3) H100-R5/28 = anti-K^k, (4) H117 = B6 anti-CBA anti-serum.

Insulin binding to different cells.

We analysed whether the I-A^k molecules on CA14 cells were responsible for the differential expression of PIns *versus* SIns determinants. The data in table I show that L929, IT.22 and CA36 cells orient PIns and SIns in the same way as do CA14 cells. This result and the finding that anti-MHC class II mAb do not inhibit insulin binding to CA14 or CA36 cells indicate that MHC class II molecules are not involved in the observed phenomenon.

T-cell reactivity against insulin-CA14 cells.

A T-cell line with specificity against both PIns and SIns was stimulated with L cells or CA14 cells incubated with either PIns or SIns for 1-18 h at 37°C. Results in table II show that a 2-h incubation period of insulin and CA14 cells was enough for efficient insulin presentation, whereas the T-cell lines were not stimulated by L cells and insulin. Activation of T cells by insulin-CA14 cells was inhibited by anti-I-A^k mAb and by anti-CD4 mAb.

A search for mAb against insulin-Ia complexes.

As the anti-Ins mAb used here are directed against native conformational determinants, they may not recognize insulin fragment/Ia complexes.

TABLE II. — **Antibody and T-cell interaction with insulin-pulsed CA14 cells.**

APC	Inc. time	P/SIns-1 T-cell proliferation in the presence of: medium	Ox6	H129	ELISA OD_{492} OxI-002
PE/Spl-SIns	—	39.371	8.035	3.391	ND
PE/Spl-PIns	—	18.352	3.956	4.477	ND
CA 14-SIns	2 h	18.891	3.055	4.124	0.087
CA 14-PIns	2 h	10.083	2.889	2.573	0.109
CA 14-SIns	6 h	23.578	5.344	5.127	0.134
CA 14-PIns	6 h	15.731	3.807	4.015	0.209
CA 14-SIns	18 h	28.343	4.881	4.811	1.534
CA 14-PIns	18 h	17.558	3.557	4.553	1.497
L929-SIns	18 h	1.057	983	954	1.337
L929-PIns	18 h	571	874	761	1.503

PE/Spl: mixture of PE and spleen cells were mitomycin-C-treated (50 μg/ml) and insulins (50 μg/ml) were present during the 4-day culture period (last 24 h in the presence of ^{3}H-thymidine). CA 14 or L cells were pulsed at 37°C with insulin 1 hour less than indicated, and mitomycin-C (100 μg/ml) added for 1 h. Then they were washed and used as APC. Data are proliferative responses of 5×10^4 responding T cells from line P/SIns-1. An aliquot of the insulin-pulsed APC were controlled for reactivity with anti-Ins mAb = OxI-002.

TABLE III. — **No evidence for insulin-Ia complex-specific antibodies.**

Mice	Inhibitor	OD_{492} values from ELISA 4 weeks	3 months	6 months	18 months
C_3H/Sscl	−	0.869	0.931	1.156	1.236
	+	0.079	0.119	0.257	0.155
B10.BR	−	0.534	0.832	1.657	1.334
	+	0.152	0.279	0.199	0.089
B10.A	−	0.788	0.781	0.993	1.003
	+	0.177	0.203	0.189	0.081
B10.A (4R)	−	0.489	0.934	1.338	0.972
	+	0.188	0.237	0.202	0.131

Groups of 20 mice were immunized with 50 μg SIns in CFA and boosted every month with SIns in IFA intraperitoneally and subcutaneously, alternatively. Bleedings were performed 7 days after injection. Antisera were titrated in cellular ELISA against SIns-CA14 cells (the present data) or SIns-CA36 cells (not shown). Values are from individual mice (representative of the group); antisera were diluted 1/20 (4 weeks), 1/100 (3 months) or 1/200 (6 or 16 months); NMS diluted 1/20-1/100 gave ELISA values of 0.050-0.120.

Inhibitor: 10^{-4} M SIns + antiserum dilutions were incubated for 24 h at 37°C before ELISA. The same results were obtained when anti-SIns antisera were incubated for only 30 min with 10^{-4} M SIns before ELISA.

Therefore, we screened antisera from about 300 mice hyperimmunized against SIns for more than a year. The antisera pretreated with insulin were analysed against SIns-pulsed CA14 or CA36 cells in the presence of free insulin, *i.e.* an assay system that would favour detection of antibodies specific for insulin-Ia complexes. In none of more than 1,500 bleedings did we find any sign of antibodies specific for insulin-Ia complexes (table III). In addition, we found that the different antisera did not inhibit T-cell proliferation induced by SIns-pulsed CA14 cells (not shown).

DISCUSSION

Our basic finding was that a panel of 18 anti-insulin mAb directed towards different common epitopes on insulin molecules reacted differently with PIns or SIns incubated with MHC class II gene-transfected fibroblasts (table I). Lack of reactivity of a given mAb (*e.g.* OxI-004 against PIns-CA14 cells but not against SIns-CA14 cells) could be due to (1) processing of insulin molecules with subsequent differential loss of that particular epitope; (2) processed insulin bound to a membrane molecule that blocks the particular epitope on PIns but not on SIns. The following membrane molecules were apparently not involved: MHC class II molecules (table I), Fc receptors (not shown), MHC class I molecules (table I and the fact that anti-MHC class I mAb had only moderate effects on insulin binding to fibroblasts) or insulin receptors (there are only about 1,000 insulin receptors/fibroblast). The fact that the

OxI-004 epitope of SIns but not of PIns (and vice versa for the HUI-018 epitope) is intact after 18-h incubation at 37°C with L929, IT.22, CA14 or CA36 cells may indicate either (1) that the enzymatic machinery in all four cell types cleaves SIns or PIns differently and this despite the fact that SIns and PIns differ only in three amino acids in the α-loop of the A-chain; or (2) that «peptide» binding membrane molecules [**4, 9, 12**] can present processed insulin differentially on the cell surface.

The smallest version of processed insulin recognized by T cells seems to be A1-A14/B7-B15 [**14**]. This molecule presented on the cell membrane may contain the OxI-004 epitope, as this mAb reacts with insulin-des-B23-30, oxidized α-chains [**21**] and lightly trinitrophenylated insulin (TNP coupled to amino acid B29) (not shown). The fine specificity of HUI-018 mAb is not known, except that most reactivity with insulin is lost after trinitrophenylation (not shown). The biological significance of the observed phenomenon is unknown: a 2-h incubation at 37°C of insulin with CA 14 cells was sufficient for formation of insulin-MHC class II complexes capable of activating T-cell proliferation, whereas approximately 18 h were needed for antibody recognition (table II). Thus, either T cells and mAb recognize different forms of processed insulin or T cells need less membrane-bound, processed insulin than can be detected by mAb in our assay (≃ 2,000 molecules/cell). However, our results demonstrate that processed insulin can be recycled to the outer membrane in the absence of MHC class II molecules.

Continued immunization with antigens leads to formation of antisera, which are more and more cross-reactive with other antigens [**3, 16**]. We thought that this might be due to synthesis of antibodies against more and more degraded antigen: hyperimmunization leads to induction of B cells, which could produce anti-peptide antibodies or MHC-restricted antibodies [**5, 6, 19, 20**]. However, by analysis of more than 1,500 antiserum samples taken at different times after immunization with insulin, we did not find any antibodies which could be classified as anti-peptide or MHC-restricted antibodies; that is, which reacted with insulin-pulsed CA14 or CA36 cells in excess of free insulin. As insulin-pulsed CA14 cells activate T cells, anti-peptide or MHC-restricted antibodies, if they are produced at all, may be directed against insulin-Ia complexes present in less than 2,000 complexes per cell.

Thus, within the limits of present technology, we may have to conclude that functional antibody and T-cell recognition is very different.

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Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1–MAGE-A1 from a nonimmunized phage-Fab library

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Antitumor antibodies with the same specificity as cytotoxic T lymphocytes that recognize antigenic peptides encoded by tumor-associated genes and presented by MHC class I molecules would be valuable tools to analyze the antigenicity or target tumor cells *in vivo*. To obtain a human antibody directed against a peptide encoded by gene melanoma-associated antigen (MAGE)-A1 and presented by HLA-A1 molecules, we selected a large phage Fab antibody repertoire on a recombinant version of the complex HLA-A1–MAGE-A1 produced by *in vitro* refolding. One of the selected phage antibodies shows binding to HLA-A1 complexed with the MAGE-A1 peptide, but does not show binding to HLA-A1 complexed with a peptide encoded by gene MAGE-A3 and differing from the MAGE-A1 peptide by only three residues. Phages carrying this recombinant antibody bind to HLA-A1$^+$ cells only after *in vitro* loading with MAGE-A1 peptide. These results indicate that nonimmunized phage Fab libraries are a source of antibodies with a T cell antigen receptor-like specificity. The human anti-HLA-A1–MAGE-A1 antibody described here may prove very useful for monitoring the cell surface expression of these complexes, and eventually, as a targeting reagent for the specific immunotherapy of HLA-A1 patients bearing a MAGE-A1-positive tumor.

phage display | major histocompatibility complex | T cell antigen receptor-like antibodies | tumor targeting

The past few years have seen the resurgence of a strong interest in tumor vaccine development (1). This is in part attributable to an increased understanding of the immune response to tumors, especially in the case of melanomas. It is now well established that human melanoma cells often express antigens that are recognized by cytotoxic T lymphocytes (CTL) derived from tumor-bearing patients. These CTLs have been used as tools to identify genes that code for tumor antigens, such as those of the melanoma-associated antigen (MAGE) gene family, which includes at least 17 related genes, namely MAGE-A1 to A12, MAGE-B1 to B4, and MAGE-C1 (2). The MAGE genes are expressed by tumors of various histological types, but they are silent in normal cells, with the exception of male germ-line cells that do not carry HLA class I molecules and are therefore unable to present antigens to CTL. Hence, antigens encoded by MAGE-A, -B, or -C genes should be strictly tumor specific. Because the MAGE antigens are shared by many tumors and on account of their strict tumor specificity, they are of particular interest for cancer immunotherapy.

Gene MAGE-A1 was isolated because it encoded an antigen presented on HLA-A1 molecules to autologous CTL of a melanoma patient (3). It is frequently expressed in metastatic melanomas (48%), esophageal squamous cell carcinomas (53%), head and neck squamous cell carcinomas (28%), non-small cell lung carcinomas (49%), or the bladder carcinomas (22%). The antigenic peptide presented by HLA-A1 molecules is EADPTGHSY (4). Several other MAGE-A1 epitopes recognized by CTL have been identified. These are presented by HLA-A3, -A24, -A28, -B53, -Cw2, -Cw3, and -Cw16 (5).

Although there is ample evidence for the presence of these antigens on a variety of tumors, they are seemingly unable to elicit an adequate antitumor immune response. Many modern cancer immunotherapies are therefore designed to induce or enhance T cell reactivity against tumor antigens. Clinical trials involving therapeutic vaccination of cancer patients with antigenic peptides or proteins are in progress (4). In a recently completed trial, 25 tumor-bearing HLA-A1 melanoma patients with advanced disease received three s.c. injections of a MAGE-A3 peptide presented by HLA-A1 (6). Tumor regression was observed in seven patients; three of these were complete. No increase in anti-MAGE CTL could be detected in the blood of these patients, including those with tumor regression. These regressions occurred very slowly, suggesting that they may have been caused by a weak immune response. To explain how these tumor regressions are obtained, and why the majority of patients do not appear to respond to the vaccines, one wishes to have precise information about the presence of the target tumor antigen on the tumor cell surface, before and after the vaccination. Currently, expression of MAGE-A and HLA class I genes is assessed with reverse transcription–PCR on tumor samples, or by immunological detection of certain MAGE-A proteins in tumor cells (7), by using a mAb such as 57B that detects MAGE-A4 in tissues, and with mAb W6/32HL that detects the presence of mature HLA class I molecules. However, positive results from these assays do not imply display of the antigenic complex. Multiple protein interactions are required for efficient assembly of MHC class I heavy chain and β2 microglobulin (β2m) with endogenous peptides (8). Peptide processing and transport into the endoplasmic reticulum involves the transporters associated with antigen processing, (TAP-1 and TAP-2), and the proteasome complex, which also includes the MHC-encoded low molecular weight proteins LMP-2 and LMP-7 (8). Loss of expression or down-regulation of any of these proteins may allow tumor cells to escape recognition by CD8$^+$ CTLs (9). Similarly, it has been shown that a mutation in the β2m gene is a frequent event leading to the loss of HLA class I surface expression in melanomas (10). Thus, a direct visualization of the HLA-A1–MAGE-A1 complex on the tumor cell surface would be the ideal way to ensure its presence. Soluble T cell receptors

Abbreviations: MAGE, melanoma-associated antigen; CTL, cytotoxic T lymphocyte; TAP, transporter associated with antigen processing; TCR, T cell antigen receptor; EBV, Epstein–Barr virus; APC, antigen-presenting cell.

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would be ideal for this purpose. Unfortunately, it has been proven difficult to engineer these molecules (11) and their inherent low affinity for their target may limit their use as detection reagents. Antibodies that specifically recognize a peptide–MHC complex have already been used to study MHC class I or II antigen presentation (12–18), to localize and quantify antigen-presenting cells (APC) displaying a T cell epitope (13, 19–21), specifically mask an autoimmune T cell epitope (22, 23), or as a targeting tool in a mouse model (24). However, selecting such reagents remains a difficult task and several failures have been reported (25, 26). The available antibodies have been obtained after immunization of mice with recombinant peptide–MHC complexes or peptide-loaded TAP-deficient APC, and recently by selection from phage-antibody libraries made from immunized transgenic mice (27). Immunization with such complexes is extremely time-consuming. Moreover, all these antibodies are of murine origin, and cannot be used repetitively in patients because of the likely development of a human anti-mouse antibody response.

We report here the selection of a fully human Fab fragment directed against the HLA-A1–MAGE-A1 complex by selection from a large nonimmune phage-antibody repertoire.

Materials and Methods

Cloning of HLA-A1 Heavy Chain. The cDNA of the HLA-A*0101 allele was amplified by PCR with the primers 5′-GCGGCG-GCGG*CCATGG*GCTCCCACTCCATGAGG-3′ and 5′-CG-GCAGGAGA*GCGGCCGC*GAGCTCCCATCTCAGGG-3′ (Eurogentec, Seraing), containing the underlined *NcoI* and *NotI* restriction sites, respectively. The PCR products were ethanol precipitated, digested with *NcoI* and *NotI* enzymes, gel purified, and ligated into the plasmid pET21d (Novagen) digested with the same enzymes. The constructs were transformed into *Escherichia coli* strain DH5α and some clones containing an insert were sequenced. Clones with a correct sequence were then transformed into *E. coli* strain BL21DE3 for production. The plasmid pHNβ2m was used to produce the β2m (28), also in *E. coli*.

***In Vitro* Refolding of the Peptide–MHC Complexes.** The peptide–MHC complexes were *in vitro* refolded from inclusion bodies produced in *E. coli* as described (28).

Biotinylation of the Refolded Complex. Centricon-10 units were used to exchange the buffer for 50 mM $NaHCO_3$ and concentrate the complex to 1 mg/ml. EZ-link sulfo NHS-SS biotin (0.01 volume of 10 mM; Pierce) was added (a final concentration of 100 μM corresponds to a 5:1 ratio of biotin:complex) and the solution was incubated for 30 min at room temperature. The biotinylated complex was separated from the free biotin by gel filtration on a Superdex 200 column (Amersham Pharmacia).

Monoclonal Antibodies. The mAb W6/32HL (anti-HLA class I heavy chain/β_2m complexes), W6/32HK (inactive variant of W6/32.HL), HB28 (BBM1) (anti-human β_2m), HC-A2 (anti-HLA-A heavy chains), and TÜ155 (anti-HLA-A/β_2m complexes with peptide-dependent reactivity) have been described (29–31). TÜ114 (IgM_κ) was produced by standard techniques and recognizes HLA-A–β_2m complexes independent of the presence of peptide in the binding groove.

Selection of Phage-Antibodies on Biotinylated Complexes. A large human Fab library containing 3.7×10^{10} antibody fragments was used for the selection (32). Phages (10^{13}) were first preincubated 1 h at room temperature in 2% nonfat dry milk-PBS in an immunotube coated with streptavidin (10 μg/ml) to deplete for streptavidin binders. Streptavidin–coated paramagnetic beads (200 μl; Dynal, Oslo) were also incubated in 2% milk-PBS for 1 h at room temperature. Phages were subsequently incubated for 1 h with decreasing amounts of biotinylated complexes (500, 100, 20, and 4 nM for rounds 1–4, respectively). Streptavidin beads were added, and the mixture was left for 15 min on a rotating wheel. After 15 washes with 0.1% Tween-PBS, bound phages were eluted by a 10-min incubation with 60 μl of 50 mM DTT, thus breaking the disulfide bond in between the complex and the biotin. The eluted phages were diluted in PBS to 1 ml and 0.5 ml were used to infect *E. coli* strain TG1 cells grown to the logarithmic phase (OD_{600} of 0.5). The infected cells were plated for amplification as described (33). After infection of TG1 cells for 30 min at 37°C, bacteria were grown overnight at 30°C on agar plates.

The diversity of the selected antibodies was determined by means of DNA fingerprinting (34). The insert of different clones was amplified by PCR with primers pUC-reverse (5′-AGCGGATAACAATTTCACACAGG-3′) and fd-tet-seq24 (5′-TTTGTCGTCTTTCCAGACGTTAGT-3′) and digested with the enzyme *Bst*NI before analysis on agarose gel.

Phage ELISA. Specificity of individual Fab fragments was assessed by ELISA with indirectly coated complexes as described (35).

Flow Cytometry Analysis on Peptide-Loaded Cells. The Epstein–Barr virus (EBV)-transformed B cell lines MZ2 (A1, A29, B37, B44, Cw6, and Cw16), LG2 (A24, A32, B35, B44, and Cw6), and AVL3 (A1, A2, B27, B44, Cw5, and Cw7) or melanoma cell lines MZ2-MEL 3.0 and MZ2-MEL 2.2 (3), were stained to demonstrate the ability of fd-Fab-G8 to bind the native HLA-A1–MAGE-A1 complex. About 10^6 cells were used for each experiment. B cells were washed twice in PBS, incubated for 30 min at 37°C in PBS containing 100 μM peptide, and then washed twice again with ice-cold 2% milk-PBS. Melanoma cells were directly resuspended in 2% milk-PBS. All subsequent washes and incubations were done in ice-cold 2% milk-PBS. Cells were incubated for 1 h at 4°C with phage-antibodies (1×10^{10} cfu) in 100 μl, washed three times, incubated with 100 μl of goat anti-fd polyclonal antibody (diluted 1/500), washed three times again and finally incubated with 100 μl of FITC-conjugated rabbit anti-goat antibody (Dako; diluted 1/50). After three washes, cells were resuspended in 500 μl of ice-cold PBS. Detection of fluorescent cells was performed by means of flow cytometry on a FACScalibur (Becton Dickinson) and the results were analyzed with the CELLQUEST program (Becton Dickinson).

Measurements of Fab–Antigens Interaction by BIAcore Biosensor. Fab-G8 was purified from *E. coli* periplasmic fraction as already described (32). Kinetic measurements were performed by surface plasmon resonance. PBS (pH 8)/0.05% Tween20 was chosen as running buffer. An NTA-chip (Amersham Pharmacia) was activated with 500 μM $NiCl_2$ for 1 min at 10 μl/min. Approximately 800 response units of hexahistidine-tagged Fab (20 μg/ml) was immobilized and the peptide–MHC complexes were subsequently injected at a flow rate of 20 μl/min to minimize rebinding effects. A blank (injection of the antibody only) was subtracted to each curve to take in account the slightly decreasing baseline caused by the Fab dissociation. The channel was regenerated by injection of 250 mM EDTA during 2 min (36).

Results

Production of HLA-A1-β2m–Peptide Complex. HLA-A1 heavy chains and β2m were produced as inclusion bodies in an *E. coli* expression system. The yields of purified inclusion bodies were 25 and 36 mg/l for the heavy chain and β2m, respectively. Recombinant products of the expected sizes (33 and 12 kDa for HLA-A1 and β2m, respectively) were visualized with SDS/PAGE analysis (Fig. 1*A*). An additional band, presumably

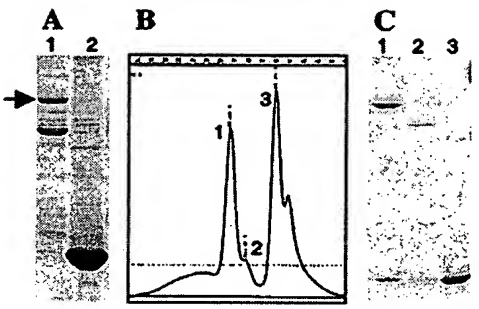

Fig. 1. (*A*) Analysis of purified inclusion bodies by SDS/PAGE and Coomassie staining. Two microliters of HLA-A1 (lane 1) or β2m (lane 2) inclusion bodies in freezing buffer were loaded on the gel. The expected size of the full-length heavy chain product is indicated with an arrow. (*B*) Gel filtration profile of the folding mixture, after an incubation of 36 h at 4°C. The mixture was concentrated by ultracentrifugation and loaded on a Superdex 200 column. The shoulder after peak 3 is a component of the folding buffer. (*C*) SDS/PAGE analysis and Coomassie staining of the peaks obtained by gel filtration. Lanes 1–3 correspond to 20 μl of fractions from peaks 1–3, respectively.

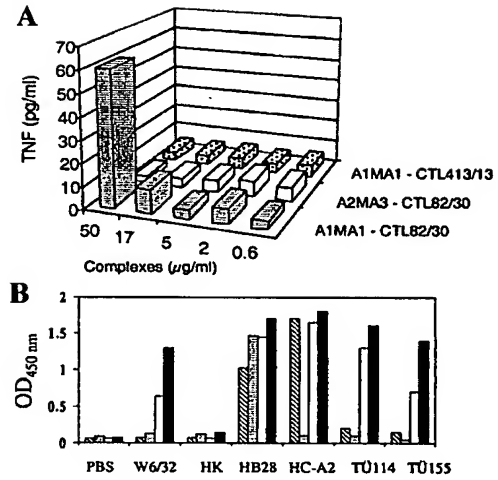

Fig. 2. (*A*) T cell activation assay with the recombinant HLA-A1–MAGE-A1 complexes. Microwells were coated with the indicated concentrations of HLA-A1–MAGE-A1 (A1MA1) or HLA-A2–MAGE-A3 (A2MA3) complexes, and washed. CTL clones 82/30 (anti-HLA-A1–MAGE-A1) or 413/13 (against a peptide presented on HLA-A2 molecules) were added at 3,000 cells per well. After 24 h, the concentration of TNF present in the culture medium was measured by testing its cytotoxicity on the TNF-sensitive WEHI-164c13 cells. (*B*) ELISA with anti-HLA mAbs. Equivalent amounts of proteins were coated directly on plastic (striped, aggregates; gray, β2m; and white, complex) or on streptavidin-coated plastic (black, biotinylated complex) and binding of several mAbs was tested. PBS, no antibody; W6/32, mAb W6/32HL binding to heavy chain–β2m dimers; HK, inactive mutant of W6/32HK; HB28, mAb binding to β2m; HC-A2, mAb binding to a nonconformational epitope of HLA molecules; and TÜ114 and TÜ155, two conformation-sensitive mAbs.

corresponding to a degradation product, was detected in the heavy chain preparation. However, this did not seem to interfere with further experiments. The purified inclusion bodies were dissolved in urea buffer. The heavy chain and β2m solutions were then diluted in a folding buffer, in the presence of the MAGE-A1 peptide, and the complexes were allowed to fold over 36 h at 4°C. The mixture was submitted to gel filtration and three major peaks were observed (Fig. 1*B*). SDS/PAGE analysis indicated that the first peak, with a molecular weight of ≈48 kDa, contained HLA-A1 heavy chain and β2m (Fig. 1*C*). This peak was absent from a control folding experiment conducted in the absence of antigenic peptide. We concluded that it corresponded to HLA-A1–β2 m–MAGE-A1 peptide complexes. The second peak corresponded to β-lactamase and the third peak was composed of free β2m. Under these conditions, up to 1.2 mg of purified complexes could be obtained from a refolding experiment by using 6 mg of heavy chain, 5 mg of β2m, and 2 mg of peptide. Thus, the observed yield was ≈10%.

Assessment of Correct Folding of the Recombinant Complexes. We verified that the recombinant complex could stimulate CTL clone 82/30, which specifically recognizes the MAGE-A1 peptide (EADPTGHSY) presented by HLA-A1 molecules (37). The CTL clone produced tumor necrosis factor when incubated in microwells coated with the HLA-A1–MAGE-A1 complex (Fig. 2*A*). No production of tumor necrosis factor was observed when CTL 82/30 was incubated with a recombinant HLA-A2–MAGE-A3 complex produced and purified with the same methods as for the HLA-A1–MAGE-A1 complexes. The HLA-A1–MAGE-A1 complexes did not stimulate another CTL clone that recognizes a peptide presented by HLA-A2 molecules. We concluded that at least a fraction of the HLA-A1–MAGE-A1 complexes were folded in such a way that they could bind the T cell receptors displayed by the specific CTL clone.

We decided to biotinylate the complexes to avoid a possible conformational change of the molecules caused by passive absorption onto plastic during phage library selection procedures. The coupling reagent was chosen to possess a disulfide bond between the reactive group and the biotin. This allowed for easy separation of the complex with bound phage-antibody from biotin-streptavidin particles by using reducing conditions. Biotinylated complexes were added to streptavidin-coated wells and their recognition by a panel of anti-HLA mAb was compared with aggregates and β2m (Fig. 2*B*). They were recognized by antibody W6/32HL which recognizes heavy chain-β2m dimers (29), the anti-β2m antibody HB28, antibody HC-A2 which recognizes a nonconformational epitope on HLA class I molecules (30), and two conformation-sensitive antibodies recognizing HLA-A–β2m complexes either in the presence (TÜ155) or also absence (TÜ114) of a peptide in the groove (31). Conversely, the aggregates were only recognized by HC-A2 and HB28. These results confirmed the correct conformation of the biotinylated complexes.

Selection of Recombinant Anti-HLA-A1–MAGE-A1 Antibodies from a Phage Display Library. In preliminary experiments, phage displaying a large repertoire (3.7×10^{10} of human recombinant Fab fragments) (32) were incubated with the biotinylated complexes and subsequently incubated with streptavidin-coated beads. To avoid the selection of antistreptavidin antibodies, the phage population was preincubated on streptavidin-coated immunotubes before selection and the bound phages were eluted with DTT. Breaking the disulfide bond between the biotin and the HLA-A1–MAGE-A1 complexes prevented the retrieval of phage bound to streptavidin. A 117-fold enrichment was obtained after four rounds of this selection procedure, and 92 clones out of 94 were binding to the HLA-A1–MAGE-A1 complexes. Without these precautions, only streptavidin-binding phage antibodies were isolated (data not shown).

The diversity of the selected antibodies was assessed by means of DNA fingerprinting, identifying 14 different patterns. The fine specificity of clones representative of each pattern was analyzed by ELISA on wells coated with HLA-A1 complexes containing either the MAGE-A1 (EADPTGHSY) or the MAGE-A3 (E*V*DP*I*GH*L*Y) peptides, which differ by only three residues (Fig. 3). For most antibodies (11/14), no difference was

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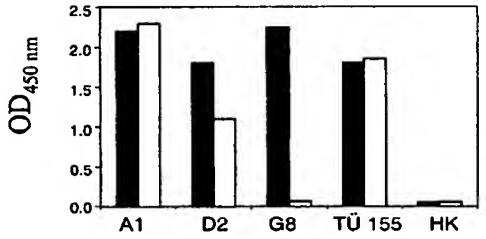

Fig. 3. Examples of specificity of recombinant antibodies selected for binding to HLA-A1–MAGE-A1 complexes. Microwells were coated with biotinylated BSA, washed, incubated with streptavidin, washed, and incubated with biotinylated HLA-A1–MAGE-A1 (black bars) or HLA-A1–MAGE-A3 (white bars) complexes. Suspensions of phages displaying the recombinant Fab fragments were then added. After washing, bound phages were detected with a mAb recognizing the p8 protein. Positive and negative controls were mAbs TÜ155 and W6/32HK (HK), respectively.

observed for the binding assay to either of the two peptides. Two antibodies, such as clone D2, appeared to bind slightly better to the complexes containing the MAGE-A1 peptide: One recombinant antibody, G8, bound to HLA-A1–MAGE-A1 but not at all to HLA-A1–MAGE-A3 complexes.

Characterization of Recombinant Fab Fragment G8. The soluble Fab fragment G8 was purified by metal affinity chromatography from the periplasm of *E. coli* by using the hexahistidine tag fused to its CH1 domain (32). Approximately 1 mg of pure material could be obtained from 1 liter of culture medium. The specificity of Fab-G8 was analyzed by surface plasmon resonance in a BIAcore instrument. In a classical-binding experiment, with the antigen immobilized on the chip, we could not find conditions that eluted the antibody without also dissociating the β2m from the HLA-A1 heavy chain. We resorted to immobilizing antibody Fab-G8 through its hexahistidine tag, and running the complexes in the soluble phase. These experiments confirmed that Fab-G8 bound to HLA-A1–MAGE-A1 but not to HLA-A1–MAGE-A3 complexes (Fig. 4). Binding to HLA-A1–MAGE-A1 occurred with on- and off-rates of 1.8×10^5 $M^{-1}\cdot s^{-1}$ and 0.045 s^{-1}, respectively, resulting in a K_D (k_{off}/k_{on}) of 250 nM.

Binding of fd-Fab-G8 to Cells Carrying the HLA-A1–MAGE-1 Antigen. Preliminary experiments indicated that a binding of the purified Fab-G8 could not be visualized on HLA-A1 cells incubated with the MAGE-A1 peptide. This was not surprising, considering the low affinity of the recombinant antibody and the low antigen density on the cell surface. To increase the avidity of the interaction, we recloned the Fab-G8 gene for display on fd

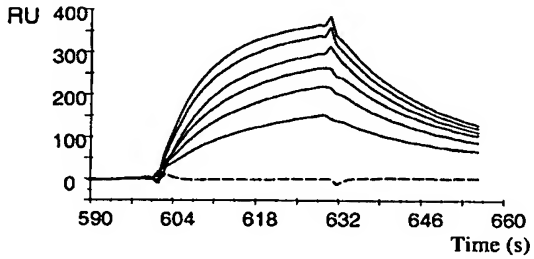

Fig. 4. BIAcore analysis of Fab fragment G8. Purified Fab-G8 was immobilized on an NTA-chip after $NiCl_2$ activation and the HLA-A1–peptide complexes were run in solution. Full lines: HLA-A1–MAGE-A1 complexes (top to bottom: 625, 542, 458, 375, 292, and 208 nM). Broken line: HLA-A1–MAGE-A3 complexes (625 nM).

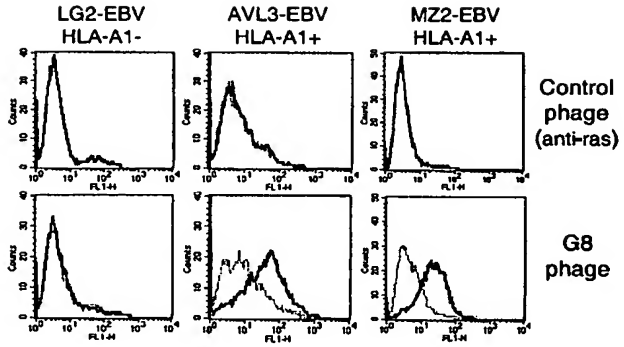

Fig. 5. Labeling EBV-transformed B cells with phage-antibody fd-Fab-G8. Cells were incubated with 100 μM peptide MAGE-1-A1 (thick lines) or MAGE-3-A1 (thin lines), and labeled with fd-Fab-G8 or a control fd phage. Bound phages were detected by sequential incubations with polyclonal goat anti-fd antibodies and rabbit anti-goat Ig antibodies coupled to fluorescein.

phage. This expression system does not need a helper phage and the resulting avidity is therefore higher than with the phagemid system in which the antibody–gp3 fusion product competes with wild-type gp3 during assembly of the capsid.

EBV-transformed B cells that did or did not express the HLA-A1 gene were incubated first with the MAGE-A1 or MAGE-A3 peptides. These cell lines are TAP^+. Consequently, only a minor fraction of HLA molecules can be loaded by this method, whereas the vast majority still displays endogenous peptides. The cells were washed, and incubated further with a suspension of fd-Fab-G8 phage. Bound phages were detected by sequential incubations with polyclonal goat anti-fd antibodies and rabbit anti-goat Ig antibodies coupled to fluorescein (Fig. 5). For the two HLA-A1 EBV-B cell lines that were tested, a shift in fluorescence intensity was observed when the cells were incubated with the MAGE-A1 but not with the MAGE-A3 peptide or when the cells were used without loading (data not shown). No shift was observed with a fd phage displaying an anti-ras Fab antibody. Furthermore, no shift in fluorescence intensity was observed when fd-Fab-G8 was incubated with an HLA-$A1^-$ EBV-B cell line, loaded with MAGE-A1 or MAGE-A3. These results demonstrate that Fab-G8 specifically recognizes HLA-A1–MAGE-A1 antigenic complexes *in situ*.

Binding of fd-Fab-G8 to (Unloaded) Melanoma Cell Lines. To confirm that Fab-G8 was capable of binding endogenously generated peptide–MHC complexes that might be present at a much lower density on the cell surface, we repeated the flow cytometry experiments with HLA-$A1^+$ and MAGE-$A1^-$ or MAGE-$A1^+$

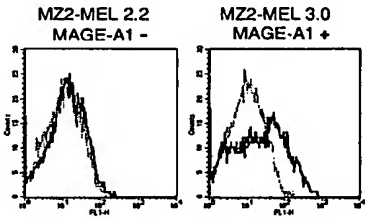

Fig. 6. Labeling melanoma cell lines with phage-antibody fd-Fab-G8. Melanoma cells MZ2-MEL 3.0 (HLA-$A1^+$, MAGE-$A1^+$) or MZ2-MEL 2.2 (HLA-$A1^+$, MAGE-$A1^-$) were incubated with phage antibodies fd-Fab-G8 (thick lines) or control phage antibodies (thin lines). Bound phages were detected by sequential incubations with polyclonal goat anti-fd antibodies and rabbit anti-goat Ig antibodies coupled to fluorescein.

melanoma cell lines. fd-Fab-G8 gave the same fluorescence intensity compared with the control on HLA-A1$^+$, MAGE-A1$^-$ cells but a higher intensity was obtained for fd-Fab-G8 compared with the control when a MAGE-A1$^+$ cell line was used (Fig. 6). The fluorescence shift was moderate compared with *in vitro*-loaded B cells, but this has to be expected because melanoma cells are expressing a lower density of HLA molecules and natural antigen processing is thought to produce a low density of HLA-A1–MAGE-A1 complexes on the cell surface. These results show that fd-Fab-G8 is capable of binding to cells which express the MHC–peptide complex at a density most likely to be found on MAGE-A1-expressing tumor cells.

Discussion

In this work, we have chosen to employ a large nonimmunized repertoire of human Fab fragments (32) to directly select human reagents with fine specificity for HLA class I–MAGE-A1, a well-characterized tumor antigen already used in clinical trials (38, 39). Until now, the few approaches that have been successful in isolating such T cell antigen receptor-like antibodies have used sophisticated immunization protocols, involving the injection of recombinant complexes or TAP-deficient *in vitro* loaded APC in syngeneic or even MHC-transgenic mice (40). The obvious advantage of this technique is a strong enrichment for peptide–MHC binders, and the use of transgenic mice may be helpful in reducing the frequency of pan-MHC reactive antibodies. However, these approaches are very time-consuming and the murine origin of the selected antibodies is a major drawback to possible future therapeutic applications. Indeed, antibodies specific for this tumor-associated antigen may eventually be used as a targeting reagent to deliver toxins or cytokines (24) specifically to the tumor site. In search of a more generic method to isolate human antibodies to MHC–peptide complexes, we have explored the use of very large nonimmunized phage–antibody libraries.

We used the phage display technique to select human antibody fragments from a large nonimmune library. One of the crucial factors determining the success of this approach relates to the state of the antigen used for selection. The conformation of the antigen has to be as "natural" as possible. We tested several methods to produce a recombinant version of the complex needed for the selection, including secretion of a single-chain peptide–HLA molecule in *E. coli* periplasm and expression in *Drosophila* cells (data not shown), but only *in vitro* refolding from inclusion bodies produced in *E. coli* yielded enough correctly folded protein. Numerous HLA complexes (class I and II) have been refolded *in vitro*, including HLA-A2, -B27, -B35, -B53 -G, -E, DR2, and DRB, demonstrating the versatility of this method for HLA molecules (41–45). However, this is the first report describing the production of a recombinant HLA-A1. This recombinant complex was biotinylated to minimize conformational changes of the antigen that may occur as a consequence of passive adsorption onto plastic (46, 47).

Using direct selection *in solution* with this antigen, we could isolate 14 different antibodies binding to the complex, with one clone (G8) showing the capability to bind in a peptide-specific manner. This antibody fragment binds in ELISA to HLA-A1–MAGE-A1 but not to HLA-A1–MAGE-A3. These two peptides display only three aa differences. This extreme specificity could be confirmed by BIAcore experiments. More importantly, phages displaying G8 did not bind HLA-A1$^+$ cells unless they were *in vitro* loaded with MAGE-A1 peptide, demonstrating the absence of binding for HLA-A1 complexes loaded with endogenous peptides. MAGE-A1 and MAGE-A3 share the same main anchor residues for HLA-A1 (Asp in position 3 or P3, and Tyr in P9) and secondary anchor residue Pro in P4. Moreover, P1, P6, and P7 are also identical. The residue in P2 (Ala in MAGE-A1; Val in MAGE-A3) is probably not important for binding because the side chain is thought to be buried in the groove (48). Interestingly, the remaining residues in P5 and P8 have their side chain pointing out of the groove in a molecular model of HLA-A1–MAGE-A3 (48). MAGE-A1 has in P5 and P8 two residues (Thr and Ser) displaying hydroxyl groups that can be involved in hydrogen bonds, conversely to the residues displayed by MAGE-A3 (Ile and Leu). Therefore, the residues in P5 and P8 have a high probability of being involved in the differential binding to CTL82/30 TCR as well as the Fab antibody G8.

As shown by surface plasmon resonance studies, Fab-G8 has an affinity of 250 nM for the complex HLA-A1–MAGE-A1. This rather low affinity was not expected because the Fab fragment was selected from a very large repertoire of 3.7×10^{10} independent clones. A large number of binders directed to diverse antigens have already been selected from this repertoire, most of them having an affinity in the 5–30 nM range (32). The fact that the Fab-G8 antibody survived four rounds of selection in competition with all of the pan-reactive antibodies is surprising, but might be explained by a high expression level, inducing a high display level of the Fab-p3 fusion protein on phage. No other peptide-specific binders could be selected from the library, and a depletion step with HLA-A1–MAGE-A3 complexes did not favor the selection of MAGE-A1-specific binders. Hence, such peptide-specific binders seem to be rare in the library despite its size. It has to be kept in mind that the targeted epitope is a peptide deeply buried inside the MHC molecule. Only between 100–300 Å of peptide bound to a MHC class I molecule is actually available for direct recognition (49). Antibodies binding to proteins contact usually 800 Å of their ligand (50). Consequently, peptide specificity can be obtained only if the major interactions between the antibody and the complex are made with the peptide. The present affinity may be sufficient for staining purposes, but is most likely too weak for *in vivo* targeting purposes. The next step is thus to mature the affinity of this antibody without losing its fine specificity. This goal is difficult to reach, because the HLA-A1 chain is thought to contribute to 65–85% of the (peptide–MHC)–antibody interface. To overcome this problem, we are now performing an affinity maturation of G8 by directed randomization of complementarity determining region H3, and reselection (51). Although it is not known which residues of the antibody are involved in the peptide recognition, it is predominantly the H3 loop that dominates in the antigen interaction. Indeed, a gain of up to 18-fold in affinity without loss of peptide specificity has already been achieved (Chames *et al.*, unpublished work). Moreover, these studies may also define which residues are directly interacting with the peptide and allow for more targeted affinity maturation. Furthermore, with such antigen–antibody interaction profiles, it may be possible to build antibody libraries with a propensity to bind HLA–peptide complexes in a peptide-specific manner.

We succeeded in selecting a human antibody binding specifically to the complex HLA-A1–MAGE-A1. This antibody may now be used to detect the presence of this specific T cell epitope by flow cytometry and possibly immunohistochemistry or immunoprecipitation and should be very useful for analysis of MAGE-1-based immunotherapies. Indeed G8, as Fab or phage-Fab, may be used to check the expression of this T cell epitope on tumor cells, before and during vaccination with MAGE-A1 peptide, or APCs loaded with MAGE-A1 (39). The display efficiency of this complex at the APC surface could also be monitored after transfection with MAGE-1 gene or after *in vitro* peptide loading.

G8 is the first human antibody directed against a class I peptide–MHC complex. This presents many opportunities. This human antibody is directed against a well-characterized

and very specific human tumor marker and in principle should be an interesting candidate as targeting moiety in an immunocytokine (52), immunotoxin (24), or in a bispecific antibody (53), in particular after antibody affinity maturation. However, the main limitation for all of these applications may be the density of the specific epitope on the cell surface. Indeed, only a small fraction of the 10^4-10^5 HLA-A1 complexes displayed per cell are expected to contain the MAGE-A1 peptide. Possibly a more sensitive and selective antitumor reactivity *in vivo* could be obtained by retargeting of T cell achieved by fusion of Fab-G8 with the CD3 ζ or γ chain (54, 55). Preliminary data suggest that a fusion protein between Fab-G8 and the CD3γ chains, once transfected into human PBL, is able to redirect T cells specifically toward MAGE-A1$^+$ melanoma cells (unpublished work). As Fab-G8 already has an affinity 5- to 500-fold higher than found for TCRs, it will be very interesting to compare the behavior of these Fab-G8-displaying T cells with CTL 82/30 that harbors a natural TCR directed against the same epitope.

To conclude, this work demonstrates that very large human nonimmunized phage libraries can be used to rapidly select antibodies of exquisite TCR-like specificity and highlights the potential of such molecules for immunodiagnostic and immunotherapeutic applications.

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